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Introduction

The work proposed for this award involved using the crystal structures of the extracellular domains of the EGF receptor (EGFR) and its homologs HER2/Neu/ErbB2, HER3/ErbB3, and HER4/ErbB4, which were recently determined in my lab and elsewhere, to design a new class of inhibitors of this family of receptors. These structures had shown that ligands bind to two separate surfaces in these receptors that are normally far apart in the absence of ligand. Binding ligand (e.g. EGF) requires a large conformational change in the receptor to bring these surfaces close together, and it is this conformational change that then leads to receptor dimerization and initiation of a signaling cascade through activation of a cytoplasmic tyrosine kinase. Our idea had been to create through mutagenesis a ligand that bound more tightly to one of the binding surfaces but not to the other. Theoretically, this ligand would bind to the receptor but not induce the conformational change needed to activate the receptor and thus serve as an inhibitor of the receptor.

In the process of designing specific mutations to achieve this goal we learned that at least two companies and two academic labs were pursuing similar if not identical strategies. After much deliberation, I decided not to duplicate their efforts but rather to redirect our energy to a different project that nonetheless involves structure-based design of ErbB inhibitor and maintains the spirit of the original proposal.

The HER2/Neu/ErbB2 member of the EGF receptor family is overexpressed in 20-25% of breast cancers, and HER2 overexpression correlates with more aggressive tumors and a much poorer prognosis. Herceptin (Trastuzumab), a monoclonal antibody directed against the HER2 extracellular region, was approved by the FDA in 1998 as a therapy for HER2-overexpressing breast cancers and demonstrated that targeting HER2 activity can have a therapeutic effect in these cancers. EGF receptor family members may be targeted in at least 2 ways: antibodies against the extracellular region (e.g. Herceptin and Erbitux, which targets EGFR and is approved for the treatment of advanced colon cancer) or small molecules that specifically inhibit the cytoplasmic kinase domains of these receptors. Tarceva and Iressa, for example, are kinase inhibitors that target the EGFR kinase and have been approved for the treatment of colon cancer.

Despite clear evidence that targeting HER2 activity can be of clinical benefit in breast cancer, no specific inhibitor of the HER2 kinase is currently available. Crystal structures of kinase regions are widely recognized as invaluable aids to the design of drugs targeting specific kinases, and the crystal structure of the EGFR kinase domain both alone and complexed with two different drugs has been reported (Stamos, Sliwkowski et al. 2002; Wood, Truesdale et al. 2004).

No crystal structure for the HER2 kinase has been reported—no doubt not for lack of trying. A likely explanation for the absence of a HER2 crystal structure is that the HER2 kinase domain has proven to be poorly behaved in solution—it aggregates, which essentially undermines efforts to grow crystals of the HER2 kinase (Jan, Johnson et al. 2000).

A strength of my lab is the use of molecular biological approaches to aid crystallization and determination of difficult structures by X-ray crystallography. We have thus undertaken to determine the crystal structure of the HER2 kinase domain both alone and complexed with drugs targeting related kinases, which weakly inhibit the HER2 kinase. The aim of these studies is to provide an molecular picture of the HER2 kinase active site and aid efforts to design HER2 specific kinase inhibitors. To bolster these efforts we have begun a collaboration with the laboratory of Phil Cole, the chairman of the Pharmacology department here at Johns Hopkins, who has extensive experience designing and testing kinase inhibitors (Parang, Till et al. 2001; Hines and Cole 2004; Hines, Parang et al. 2005). In addition to commercially available kinase inhibitors that weakly inhibit the HER2 kinase, Prof. Cole's lab has developed novel inhibitors of the EGFR kinase and wishes to apply their methods to development of HER2 kinase inhibitors. We will work closely with Prof. Cole's lab to perform kinase activity and inhibition assays to access novel classes of potential HER2 kinase inhibitors.

As detailed in the next section, we have initiated a new approach using mutagenesis to create a more soluble form of the HER2 kinase, which we hope will prove more amenable to X-ray crystallographic studies. We have also created a cell line expressing a full-length form of HER2 so that the activity of native HER2 can be compared to our expressed HER2 kinase domain in assays of enzymatic function and the activity of specific inhibitors and, if sufficient intact HER2 can be purified, crystallization trials of the intact receptor may be initiated. These results are the essential first step to producing the reagents needed to pursue structural and functional studies of the HER2 kinase and potential HER2 kinase inhibitors.

Body

The aims of the current project are to: **(1)** produce a soluble form of the HER2 kinase domain for structural and enzymatic studies (the EGFR kinase domain will also be produced for comparisons and control experiments), **(2)** determine the crystal structure of the HER2 kinase domain both alone and complexed with lead inhibitor compounds that are available both commercially and in the laboratory of our collaborator Phil Cole, **(3)** perform enzymatic assays of the HER2 kinase in the presence and absence of inhibitors and modified inhibitors to aid design and selection of more potent HER2 kinase inhibitors, and **(4)** produce a full-length form of HER2 for enzymatic and structural assays and comparison with the isolated HER2 kinase domain.

Aim 1. Earlier work on the HER2 kinase domain has been bedeviled by problems with poor solubility and unstable protein (Jan, Johnson et al. 2000). These issues have not arisen for studies of the EGFR kinase domain, which is well behaved and has been crystallized by two independent groups (Stamos, Sliwkowski et al. 2002; Wood, Truesdale et al. 2004). Since the EGFR and HER2 kinase domains share 78% sequence identity, and the known structure of the EGFR kinase domain allows identification of residues exposed on the surface of the kinase, we identified surface residues that are hydrophobic in HER2 but hydrophilic in EGFR. By substituting the HER2 residues with the EGFR residues we do not expect to alter either the kinase structure or activity, but we do expect to improve the solubility properties of HER2. Appendix 1 shows an alignment of the amino-acid sequences of each of the ErbB kinase domains. Above this alignment are indicated residues that were changed in HER2 to introduce more hydrophilic or charged surface residues. We typically preferred changes to Asp instead of Glu so as not to introduce "high rotamer" side chains and to introduce a charged side chain, which is likely to promote solubility more than non-charged hydrophilic residues.

These substitutions have been successfully introduced into the HER2 kinase domain, and this variant form of the HER2 kinase domain (solHER2KD) expressed using a baculovirus expression system. We have also concurrently expressed the native HER2 kinase domain (HER2KD) and the native EGFR kinase domain (HER1KD) to compare the enzymatic activities of each of these proteins (the native HER2KD will be solubilized in detergents for enzymatic assays) and ensure that the substitutions in solHER2KD have minimal effect on the kinase activity or its susceptibility to various inhibitors. Figure 1 shows a Western blot of these proteins following expression in Sf9 cells using a baculovirus system. This result demonstrates robust expression of the wild type EGFR kinase domain and the solubilized form of the HER2 kinase domain. The wild type form of the HER2 kinase domain expresses poorly, however, consistent with earlier investigator's difficulty expressing this protein.

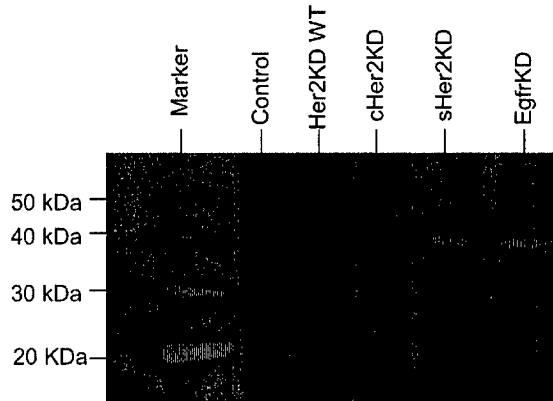


Figure 1. Anti-His-tag Western blot showing expression of wild type HER2 kinase domain (Her2KD WT), a catalytically-dead form of the HER2 kinase domain (cHer2KD), the solubilized form of the HER2 kinase domain (sHer2KD), and the wild type EGF receptor kinase domain (EgfrKD). The expected molecular weight of the kinases is 40 kD.

Attempts to purify the EGFR and solHER2 kinase domains stalled owing to inefficient binding of the expressed protein to a Nickel-NTA column (the expressed proteins have an N-terminal polyhistidine tag). To remedy this problem, each of the kinase domains has been re-engineered to contain N-terminal myc- and strept-tags for easier identification and purification, respectively (Lichty, Malecki et al. 2005). Virus for these re-engineered proteins has been produced and protein expression experiments are underway. We estimate that expression levels for solHER2KD and EGFRKD were ~1 mg/liter of cell culture, and we expect to have comparable amounts of more easily purifiable protein shortly. When this protein becomes available, we will initiate assays of enzymatic function using standard substrates available in

the Cole lab. We will also initiate crystallization studies of the solHER2KD both alone and complexed with the EGFR inhibitors Tarceva, Iressa, and a compound developed in the Cole lab, which exhibit low levels of HER2 inhibition.

Aims 2&3. Aims 2 and 3 rely on the successful purification of the solHER2KD, and work on these aims has not yet been initiated.

Aim 4. To provide an intact form of HER2, which consists of an extracellular region followed by a single transmembrane-spanning region, a cytoplasmic kinase domain, and a C-terminal tail of ~200 amino acids that becomes phosphorylated and recruits adaptor proteins upon receptor activation, we have created a cell line overexpressing HER2. To aid detection and purification of HER2, we have overexpressed HER2 as a fusion protein with human growth hormone (hGH) and a polyhistidine tag at the N-terminus. A tobacco etch virus (TEV) protease site separates the hGH and His-tag from the HER2 sequences and allows release of native HER2.

Initial attempts to produce a cell line overexpressing HER2 resulted in roughly 1/3 of the expressed protein being cleaved at the C-terminus as judged by

Western blots of cell lysates. This cleavage corresponded to loss of the C-terminal tail, which has also been observed for EGFR. Loss of this tail does not affect EGFR activity (data not shown).

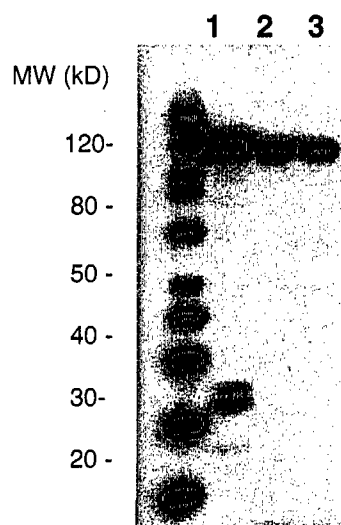


Figure 2. Anti-hGH Western blot of cell lysates from cells overexpressing (1) tEGFR, (2) clone 1 of tHER2, and (3) clone 2 of tHER2.

To enable purification of a homogenous form of HER2 for crystallization and activity studies, we introduced a stop codon immediately after the HER2 kinase coding region and re-expressed this truncated form of HER2. This truncated version of HER2 (tHER2) and a similarly truncated version of EGFR (tEGFR) were transfected into CHO Lec1 cells. Positive transformants were selected in geneticin-containing media (geneticin-resistance and dihydrofolate reductase genes were co-transfected with the receptor gene) and later amplified using methotrexate. Fluorescence-activated cell sorting (FACS) was used to isolate clonal, high-expressing cell lines. Figure 2 shows a Western blot indicating expression of tHER2 (and tEGFR) with minimal proteolysis.

These cell lines express $\sim 10^6$ receptors per cell as judged by both ELISA and Western blot using free hGH as a standard. Initial efforts to purify tEGFR revealed that it was forming disulfide-linked oligomers, presumably via the free cysteines in the cytoplasmic region. The presence of 25 conserved disulfide bonds in the extracellular regions of both EGFR and HER2 made us reluctant to add reducing agent to reduce the intermolecular disulfides for fear of also reducing the intramolecular disulfides. We have thus used site-directed mutagenesis to substitute alanine or serine for the cytoplasmic cysteines (none of which are believed to be necessary for enzymatic activity) and are in the process of selecting cell lines expressing these cytoplasmic cysteine-less variants of tEGFR and tHER2.

Key Research Accomplishments

1. Expression of a solubilized version of the HER2 kinase domain using a baculovirus expression system.
2. Expression of wild type EGFR kinase domain using a baculovirus expression system.
3. Re-engineering the EGFR and solHER2 kinase domains to simplify purification and detection.
4. Creation of cell lines overexpressing stable, active forms of transmembrane versions of EGFR and HER2.

Reportable Outcomes

None so far.

Conclusions

1. Adding solubilizing mutations to the HER2 kinase domain appears to be a successful approach to enhancing the expression and solubility of the HER2 kinase domain.
2. Truncating full-length versions of EGFR and HER2 allows overexpression of a stable and active forms of these receptors.
3. These approaches will enable isolation of sufficient amounts of HER2 and the HER2 kinase domain to perform structural and biophysical studies.

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Appendix

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HER2 SOL Subs      D                      K          D    D
HER2_human          GAMPNQAQMRILKETELRKVKVLGSGAFGTVYKGIWIPDGENVKIPVAIKVLRENTSPKA 60
EGFR_human          GEAPNQALLRILKETEFKKIKVLGSGAFGTVYKGLWIPEGEKVKIPVAIKELREATSPKA 60
HER4_human          GTAPNQAQLRILKETELRKVKVLGSGAFGTVYKGIWVPEGETVKIPVAIKILNETTGPKA 60
HER3_human          -EKANKVLARIFKETELRKLVKVLGSGVFGTVHKGVWIPEGESIKIPVCIKVIEDKSGRQS 59
                   .*: .  **:****: : : :*****.*****:**:*:*. :****.*** : : : . :

HER2 SOL Subs      D
HER2_human          NKEILDEAYVMAGVGSPIVSRLLGICLTSTVQLVTQQLMPYGCILLDHVRENRRGLGSQDLL 120
EGFR_human          NKEILDEAYVMASVDNPHVCRLLGICLTSTVQLITQQLMPFGCLLDYVREHKDNIGSQYLL 120
HER4_human          NVEFMDEALIMASMDHPLVRLGLVCLSPTIQLVTQQLMPHGCLLEYVHEHKDNIGSQLLL 120
HER3_human          FQAVTDHMLAIGSLDHAHIVRLGLCPGSSLQVLVTQYLPLGSLLDHVRQHRGALGPQLLL 119
                   . *.  : : : . : : :****:*  : :*:**:* * *.*****: : : : . :*. * **

HER2 SOL Subs      R
HER2_human          NWCMIQIAKGMSYLEDVRLVHRDLAARNVLVKSPNHVKITDFGLARLLDIDETEHADGGK 180
EGFR_human          NWCVQIAKGMNYLEDRLVHRDLAARNVLVKTPQHVKITDFGLAKLLGAEKEYHAEGGK 180
HER4_human          NWCVQIAKGMMYLEERLVHRDLAARNVLVKSPNHVKITDFGLARLLLEGDEKEYNADGGK 180
HER3_human          NWGVQIAKGMYYLEEHGMVHRNLAARNVLLKSPSQVQVADFGVADLLPPDDKQLLYSEAK 179
                   ** :***** ***: :***:*****:*:*. :*: :*****:* ** : : : . . *

HER2 SOL Subs      D
HER2_human          VPIKWMALESILRRRFTHQSDVWSYGVTVWELMTFGAKPYDGIPAREIPDLLEKGERLPQ 240
EGFR_human          VPIKWMALESILHRIYTHQSDVWSYGVTVWELMTFGSKPYDGIPASEISSILEKGERLPQ 240
HER4_human          MPIKWMALECIHYRKETHQSDVWSYGVTIWELMTFGGKPYDGIPTREIPDLLEKGERLPQ 240
HER3_human          TPIKWMALESIHFGKYTHQSDVWSYGVTVWELMTFGAEPYAGRLAEVPDLLEKGERLAQ 239
                   *****.* :*****:*****.*:*** *:  *: : :*****.*

HER2 SOL Subs      D
HER2_human          PPICTIDVYMIMVKCWMIDSECRPRFRELVSFMRMARDPQRFVVIQ-NEDLGPAS-PLD 298
EGFR_human          PPICTIDVYMIMVKCWMIDADSRPKFRELIIEFSKMARDPQRYLVIQGDERMHLPS-PTD 299
HER4_human          PPICTIDVYVMVKCWMIDADSRPKFKELAAEFMRMARDPQRYLVIQGDDRMKLPS-PND 299
HER3_human          PQICTIDVYVMVKCWMIDENIRPTFKELANEFTRMARDPPRYLVIKRESGPGIAPGPEP 299
                   * *****:***** : ** *:** **:***** *:***: : . . *

HER2 SOL Subs      D          * End of Kinase Domain
HER2_human          STFYRSLLLEDDDDMGDLVDAEEYLVPQQG-----FFCPDPAPGAGGMVHHRSSSTRSG 352
EGFR_human          SNFYRALMDEEDMDDVVDADAEEYLIPQQG-----FFS-----SPSTS-- 335
HER4_human          SKFFQNLLDEEDLEDMDAEEYLVPQAFNIPPPIYTSRARIDSNRSEIGHSPPPAYTPMS 359
HER3_human          HGLTNKKLEEVELEPELDLDLDLEAEEDNLATTTLGSALSPLVGTNLNRPRGSQSILSPSS 359
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